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Use of methanol as cryoprotectant and its effect on *sox* genes and proteins in chilled zebrafish embryos ☆

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ABSTRACT

Methanol is a widely used cryoprotectant (CPA) in cryopreservation of fish embryos, however little is known about its effect at the molecular level. This study investigated the effect of methanol on sox gene and protein expression in zebrafish embryos (50% epiboly) when they were chilled for 3 h and subsequently warmed and cultured to the hatching stages. Initial experiments were carried out to evaluate the chilling tolerance of 50% epiboly embryos which showed no significant differences in hatching rates for up to 6 h chilling in methanol (0.2-, 0.5- and 1 M). Subsequent experiments in embryos that had been chilled for 3 h in 1 M methanol and warmed and cultured up to the hatching stages found that *sox2* and *sox3* gene expression were increased significantly in hatched embryos that had been chilled compared to non-chilled controls. *Sox19a* gene expression also remained above control levels in the chilled embryos at all developmental stages tested. Whilst stable sox2 protein expression was observed between non-chilled controls and embryos chilled for 3 h with or without MeOH, a surge in sox19a protein expression was observed in embryos chilled for 3 h in the presence of 1 M MeOH compared to non-chilled controls and then returned to control levels by the hatching stage. The protective effect of MeOH was increased with increasing concentrations. Effect of methanol at molecular level during chilling was reported here first time which could add new parameter in selection of cryoprotectant while designing cryopreservation protocol.

1. Introduction

Methanol has been a widely used cryoprotectant in the cryopreservation of embryos and oocytes and other reproductive tissues. However, success of most of cryopreservation protocols is usually measured by either physical appearance of cell or survival rate. It has previously been reported that chilling alters the pattern of *sox* gene expression in zebrafish embryos [8]. Similarly, the effect of cryoprotectant use at the molecular level is still unknown. In the present study, the effect of chilling in the presence of cryoprotectant methanol on gene and subsequent protein expression was investigated.

Cryoprotectants usually protect cells from chilling and freezing injury by dehydrating cells and lowering the freezing point [35]. The use of cryoprotectants in low temperature storage has been proven to be essential in protecting cells from chilling injury [49]. However, most cryoprotectants are toxic especially when used at high concentrations [45]. The toxicity of cryoprotectants to cells is also dependent on their type, exposure temperature and exposure time period [40]. Cryoprotectants can cause cellular injury by osmotic trauma [34] and can be toxic to the cells. Cryoprotectant toxicity studies are now common practice prior to their use in cell cryopreservation. However there is very limited information on how cryoprotectants function at the molecular level and if they have a significant effect on gene or protein expression following cryopreservation. Understanding of the impact of cryoprotectants at the molecular level is important especially for reproductive materials such as embryos, oocytes, ovarian tissues. Any changes at the molecular level could have a lethal effect on subsequent development. Any alteration during these early stages could be replicated leading to long term genetic defects. Studies in mouse and rat embryos have shown that methanol (MeOH) is toxic

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[22] and even lethal when used at high concentrations (12–16 mg/mL) [2]. Methanol is a widely used cryoprotectant in fish embryo cryopreservation. Methanol has been found to protect cells during cryopreservation in zebrafish oocytes and embryos [43,49] and common carp embryos [1]. It has been found that methanol was effective in zebrafish embryo cryopreservation because it has low toxicity compare to other most commonly used cryoprotectants [49] and also due to its ability to pass through the embryo membrane rapidly [17]. Similar studies in medaka also demonstrated higher embryo survival rates after chilling in the presence of MeOH [45] than chilling in ethylene glycol (EG), propylene glycol (PG), DMSO and glycerol. However, it has also been shown that methanol exposure is associated with visual impairment or blindness, affecting the optic nerve and retina of rats at concentrations 20% w/v of 4 g/kg followed by 2 g/kg [12]. Methanol has also been demonstrated to be neurotoxic where its exposure leads to severe central nervous system (CNS) defects in mice at gastrulation periods [7] and in drosophila embryos at 8–11 embryonic stages [26]. Rico *et al.* showed that methanol also alters ecto-nucleotidases and acetylcholinesterase enzymes (important for neuromodulation in brain) in zebrafish brains [36]. Therefore it is important that the effect of methanol is better understood when used as a cryoprotectant.

The present study investigated the effect of chilling on *sox* gene and protein expression in the presence of methanol. *Sox* genes (*sox2*, *sox3* and *sox19a*) are important genes in the development of nervous systems in zebrafish embryos and any changes can lead to serious abnormalities [13]. Inhibition of *sox* gene expression in vertebrate embryos results in premature differentiation of neural precursors and their overexpression results in inhibition of neurogenesis [4,5,15,19,32]. However, study on gene expression (mRNA level) does not provide information on protein translation as the efficacy of translation can also be affected by post transcription modulation of regulatory genes [25]. It has been demonstrated that small non-protein-coding RNAs (small nucleolar RNA, micro RNAs, short interfering RNAs, small double stranded RNA) also regulate gene expression, including translation in developmental processes [25]. Therefore, following gene expression studies, subsequent protein expression studies were also carried out to understand the effect of MeOH at the molecular level during cryoprotectant exposure and chilling. For these study, embryos were chilled for up to 24 h to find out optimum chilling storage period (commonly used transportation period) using MeOH as cryoprotectant. It is also further necessary to test optimum concentration which not only improve survival rate but also does not change anything at genetic level, especially neurological genes and proteins i.e. *sox*, due to their neurotoxic tendency. Once this condition has been optimised, further cryopreservation protocol would be developed by addition of sugars without compromising genetic integrity.

2. Materials and methods

2.1. Zebrafish maintenance and embryo selection

All the procedures and protocol carried out in this study were ethically approved by LIRANS institute of Research in the Applied Natural Science's (LIRANS) Ethical Board Committee and University of Bedfordshire Ethical Scrutiny Committee. Adult zebrafish 12–14 weeks old were maintained in 40 L glass tanks at 27 ± 1 °C. The males and females were kept at a ratio of 1:2 and a 12 h light/dark cycle was used. Fish were fed 3 times a day with TetraMin® (Tetra, Germany) and once a day with freshly hatched brine shrimp (*Artemia salini*) (ZM systems, UK). Embryos were collected in the morning and kept in a 27 ± 1 °C water bath until the desired stage was reached. Embryonic stages were determined using light microscopy (Leica MZ95, Germany) according to the morphology described by Kimmel [18].

3. Experimental design

3.1. Experiment 1: Impact of chilling at 0 °C on embryo hatching rate for different time periods in the presence of MeOH as a cryoprotectant

Embryos (50% epiboly) were chilled [23] at 0 °C in crushed ice (temperature was maintained throughout by addition of ice) for up to 24 h (3-, 6-, 18- and 24-h) in the presence of different concentrations of MeOH (0.2, 0.5 and 1 M). After chilling, cryoprotectant MeOH was replaced with egg water (60 μg/mL sea salt in distilled water) and the test tubes were quickly placed into a 27 ± 1 °C water bath and embryos were then incubated at 27 ± 1 °C for up to 3 days or until they hatched. Control embryos were kept at 27 ± 1 °C and incubated for 3 days or until they hatched. Hatching rates were then monitored and all experiments were repeated 3 times in triplicate (total embryo = 2025). Embryos were considered to be hatched when their chorion is missing, there were no obvious signs of malformation, and there was natural movement with a functional heartbeat. Embryos were considered unhatched if they showed no signs of cell differentiation, yolk coagulation and no tail formation or detached tail and/or if they remained in the chorion [21].

3.2. Experiment 2: Impact of chilling and warming on sox gene expression in zebrafish (Danio rerio) embryos in the presence of MeOH

Based on the results obtained from the previous experiments, further studies were carried out on the effect of 3 h chilling on gene and protein expression in 50% epiboly embryos in the presence of MeOH.

3.2.1. Chilling of embryos

Embryos at 50% epiboly stage were chilled at 0 °C for 3 h with different concentrations of MeOH (0.2, 0.5 and 1 M) as described above. RNA was then extracted and cDNA was produced as described below [8]. cDNA was diluted 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR. For each time point, three different biological samples (5 embryos/tube) were treated and stored. Each experiment was repeated 3 times. Experimental controls were kept at 27 ± 1 °C in a water bath for the equivalent time period.

3.2.2. Warming and incubation of embryos after chilling

Embryos at 50% epiboly stages were chilled for 3 h at 0 °C, they were then warmed up and incubated at 27 ± 1 °C until three key developmental stages –20 somites stage (hind brain development), heartbeat stage (first heart beat starts) and hatching stages (first time when actual larvae exposed to environment). For each embryonic stage, three different samples (5 embryos/tube) were treated and stored for RNA extraction at –80 °C and real time PCR. Each experiment was repeated 3 times. Experimental controls were kept at 27 ± 1 °C in water bath.

3.3. Experiment 3: Impact of MeOH chilling and warming on sox2 and sox19a protein expression in zebrafish (D. rerio) embryos

Embryos (75 embryos) from 50% epiboly stages were chilled for 3 h with/without 1 M MeOH and then returned to 27 ± 1 °C and incubated until they hatched. Cryoprotectant solution was replaced by 27 ± 1 °C egg water (60 μg/mL sea salt in distilled water) following chilling before incubation. Experimental controls

were kept at 27 ± 1 °C in a water bath for the equivalent time period. Samples were collected for protein extraction immediately after 3 h chilling and larvae (after ~3 day warming).

3.4. RNA extraction and DNase treatment

RNA was extracted from embryo samples using RNAqueous Micro RNA Isolation Kit (Ambion, UK) according to the manufacturer's protocol. This protocol also includes a DNase I treatment step. RNA was stored at −80 °C until further use. RNA was checked for quantity and purity using Biophotometer (Eppendorf, UK) at 260 nm and 280 nm.

3.5. Reverse transcription

One μg of RNA was transcribed using Precision qScript Reverse Transcription Kit (Primerdesign Ltd., UK) according to the manufacturer's protocol. For the conventional PCR undiluted cDNA was used in subsequent steps. For real time PCR experiments, cDNA was diluted 1:2 in molecular biology grade water (Sigma, UK) and stored at −80 °C.

3.6. PCR analysis

The PCR reactions were consisted of NH_4 PCR buffer (Bioline, UK), 200 μM dNTP (Bioline), 1.5 mM $MgCl_2$ (Bioline), 2 U BIOTAQ™ DNA polymerase (Bioline), 0.5 μM each primer (see Table 1), 1 μg RNA template and PCR water. Standard conditions for PCR were initial denaturation at 94 °C for 5 min (1 cycle), 40 cycles of amplification contains 94 °C for 30 s, annealing temperature (see Table 1) for 30 s, 72 °C for 30 s followed by 1 cycle of additional extension step 72 °C for 10 min. The PCR products were run on 2% agarose gels and stained with ethidium bromide (0.5 μg/mL, Sigma, UK).

3.7. Generation of standards for real time PCR

The standards for real time PCR of *sox2*, *sox3* and *sox19a* along with housekeeping genes EF1-α and β actin (Lin *et al.* [23]) were produced using conventional PCR as described above. The primer sequences are given in the Table 1. DNA was isolated from excised bands using the EZNA Gel extraction kit (Omega Bio-Tek through VWR, UK) according to the manufacturer's instructions. The isolated DNA was quantified using a Biophotometer (Eppendorf, UK) at 260 nm and diluted to 2 ng/μl followed by 10-fold serial dilutions to generate standards for real time PCR.

3.8. Quantification of sox2, sox3 and sox19a using real time PCR

Real time PCR was performed on RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor to quantify the expression level of *sox2*, *sox3* and *sox19a*. Reaction tubes contained 7.5 μl of sensimix 2× reaction buffer (contained heat activated DNA polymerase, Ultrapure dNTPs, $MgCl_2$, SYBR® Green I), 333 ηm of each primer (see Table 1) and 2 μl of cDNA sample, made up to 15 μl with PCR water. The reaction conditions were 1 cycle at 95 °C for 10 min, followed by 50 cycles at 95 °C for 10 s, the appropriate annealing temperature (see Table 1) for 15 s and at 72 °C for 15 s. Data were acquired on FAM/SYBR channel at the end of each extension step. Melt curves were also analysed to check for the absence of mispriming and amplification efficiency was calculated from a standard curve (efficiencies were in ranged from 0.8 to 1.0 and R^2 from 0.99 to 1). The possibility of genomic DNA amplification was eliminated by use of primers that crossed introns. Relative gene expression levels were calculated using the two standard curve quantification method in the Rotorgene software [33]. EF1 α and β actin were used for this study as these genes were shown to have the highest stability during chilling of zebrafish embryos [23].

3.9. Protein expression analysis

3.9.1. Extraction of protein and quantification

Embryos (75 embryos/treatment) were washed twice with embryo medium 2 (EM2) (15 mM NaCl, 0.5 mM KCl, 0.27 mM $CaCl_2$, 1 mM $MgSO_4$, 0.27 mM $NaHCO_3$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4). Following washing, embryos were subjected to protease treatment (2 mg/ml, Sigma–Aldrich) for 10 min where chorion was partially digested. After digestion, loosened chorion was removed by gentle suction and friction, resulting from pipetting the embryo up and down. Embryos were then washed 3 times with EM2 before being transferred to a 1.5 ml tube. 100 μl of protein extraction buffer (0.125 M Tris–HCl, 4% SDS, 20% glycerol) was added to each tube and samples were heated to 95 °C for 10 min. Following heating, samples were vortexed and centrifuged at 13,000×*g* for 10 min and protein containing supernatant was collected. Isolated proteins were quantified using QuantiPro™ BCA Assay Kit (Sigma–Aldrich) according to the manufacturer's instructions.

3.9.2. Western blot and immunostaining

Extracted protein was separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were loaded on 4% stacking gel and separated on 10% resolving gel at 200 V for 40–60 min. Proteins were then transferred to PVDF membrane using the semi dry method (BioRad, UK). Immunoblotting was carried out using Anti rabbit WesternDot 625 Western Blot Kit (Invitrogen, W10132). The membrane was blocked in 10 ml of WesternDot blocking buffer for 1 h at room temperature. Following blocking, membranes were incubated with 10 ml of primary antibody solution (dilution 1:1000) overnight at 4 °C on gel rocker. Primary antibodies *sox2* (Abcam, Cambridge, UK), sox19a and *β-actin* (Eurogentech, Belgium) were used at 1:1000 concentration diluted in PBS. The membranes were then washed 3 times

Table 1
Information on gene name, accession ID and primer sequences including annealing temperature and product size.

Gene name	Accession ID	Forward/reverse primers	Annealing temp. (°C)	Amplicon size (bp)
sox2	NM_213118.1	F :CTCGGGAAACAACCAGAAAA R: TCGCTCTCGGACAGAAGTTT	58	171
sox3	NM_001001811.2	F: ACCGAGATTAAAAGCCCCAT R: TTGCTGATCTCCGAGTTGTG	56	182
sox19a	NM_130908.1	F: TGTCAACAGCAACAACAGCA R: GTTGTGCATTTTGGGGTTCT	57	126
EF1-α	NM_131263.1	F: CTGGAGGCCAGCTCAAACAT R: ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87
β actin	NM_181601.3	F: CGAGCTGTCTTCCCATCCA R: TCACCAACGTAGCTGTCTTTCTG	59	86

for 10 min with WesternDot Wash buffer. Following washing, the membranes were incubated with 10 ml of Biotin-XX-Goat anti-rabbit solution for 2 h at room temperature. After secondary antibody incubation, the membranes were washed 3 times as before. The membranes were then incubated with 10 ml of Qdot 625 Streptavidin conjugate solution for 1 h at room temperature before washed 3 times as previously, followed by a final wash in MilliQ water for 5 min. The membranes were soaked in 100% methanol to make it transparent and then visualised under an UV trans-illuminator with images taken.

3.10. Statistical analysis

Statistical analysis was carried out using SPSS V.16 (IBM, USA) and Microsoft Excel (Microsoft corp. USA). Densitometry analysis was carried out using ImageJ software (Maryland, USA). All protein bands were quantified and then normalised with respect to non-treated samples. Internal control β actin was used for normalisation of any variation in replicates. The one-sample Kolmogorov–Smirnov test was performed to determine whether the data for each gene/protein were normally distributed. Where the data were normally distributed, significant differences in gene/protein expression levels between fresh and chilled embryos at the same time point were calculated using the *t*-test. One way ANOVA was carried out followed by Tukey's post hoc tests to identify changes of gene/protein expression levels between treatments. Where data were not normally distributed after logarithmic transformation, the Mann–Whitney *U* test was used instead. All gene and protein expression data were presented as mean ± SEM and *p* values of less than 0.05 were considered to be significant.

4. Results

4.1. Experiment 1: Impact of chilling at 0 °C for different time periods in the presence of MeOH as a cryoprotectant on embryo hatching rates

Before embryos were subjected to molecular studies using MeOH, embryos should be able to withstand the experimental conditions without impact on hatching rate. This experiment was carried out to assess the effect of chilling on 50% epiboly stage embryos in the presence of different concentrations of MeOH up to 24 h. During this period, limited developmental changes were observed. No significant differences in hatching rates (over 85%) (Fig. 1) were found between 3 and 6 h chilled embryos. Significant decreases in embryo hatching rates were observed in embryos that were chilled for 18 and 24 h with or without MeOH. It was also observed that there were no significant differences in hatching rates between embryos chilled in MeOH and embryos chilled in egg water. Similarly, no significant differences in hatching rates were observed between embryos that were chilled with different concentrations of methanol.

4.2. Experiment 2.1: Impact of chilling and warming in the presence of MeOH on sox2 gene expression in zebrafish (D. rerio) embryos

4.2.1. Comparisons of different concentrations of MeOH on sox2 gene expression

Sox2 gene expression (Fig. 2a) in non-chilled control embryos was relatively stable throughout the tested developmental stages (from 50% epiboly stage to hatching stage). Expression of *sox2* in embryos that were chilled with or without MeOH decreased significantly when compared to non-chilled control embryos and increased following warming and culturing at 27 ± 1 °C to the non-chilled control level by the hatching stage. However, *sox2* gene expression in embryos chilled in 1 M MeOH was significantly increased when compared to non-chilled control embryos at hatching stage.

4.2.2. Comparisons of sox2 gene expression at specific stages throughout development

Sox2 gene expression (Fig. 2b) was remained stable at all tested stages in control non chilled embryos. However, significant decreases were found in embryos that were chilled at 0 °C with or without MeOH when compared to 0 h. In the embryos that were chilled in the presence of egg water and warmed at 27° ± 1 °C, expression levels returned to the similar level to time 0 by the hatching stage. Similar patterns of expression were observed in embryos that were chilled with 0.2-, 0.5- and 1 M MeOH and warmed at 27° ± 1 °C until the hatching stage.

4.3. Experiment 2.2: Impact of chilling and warming on sox3 gene expression in zebrafish (D. rerio) embryos in the presence of MeOH

4.3.1. Comparisons of different concentrations of MeOH on sox3 gene expression

In non-chilled control embryos, *sox3* (Fig. 3a) gene expression was stable until the heartbeat stage before decreasing again by the hatching stage. In embryos that were chilled with egg water and 0.2 M MeOH, significant decreases of *sox3* expression were observed immediately after chilling and after culturing at 27 ± 1 °C at 20 somites stage when compared with non-chilled controls. The expression level returned to non-chilled control

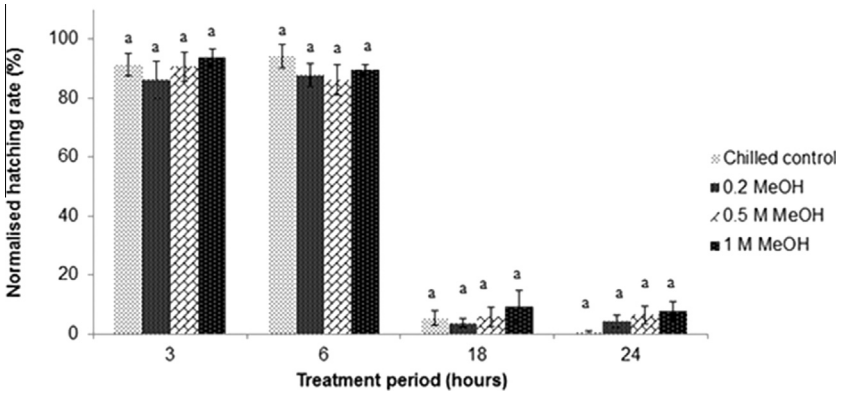

Fig. 1. Effect of chilling on hatching rates in 50% epiboly stage of zebrafish embryos: Bars represent hatching rates of zebrafish embryos after chilling at 0 °C for different time periods (3–24 h) in different concentrations of MeOH (0.2, 0.5 and 1 M), followed by incubation at 27 ± 1 °C for 3 days. Error bars represent the standard errors of the mean (SEM) (n = 9).

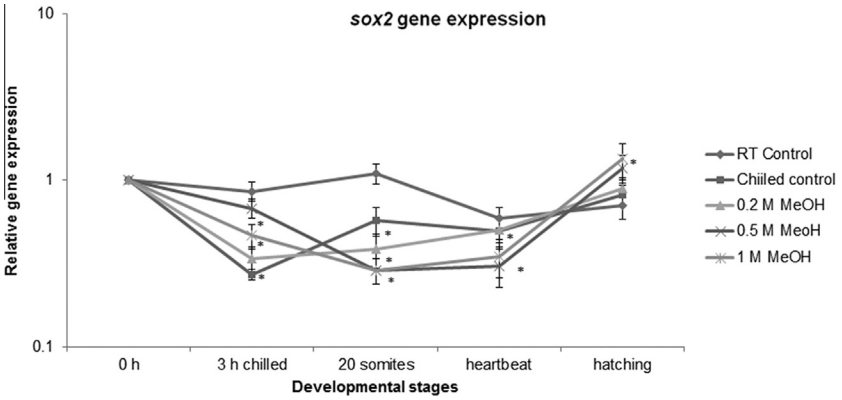

Fig. 2a. Effect of chilling in different concentrations of MeOH and warming on *sox2* gene expression in zebrafish embryos. The Figure shows the gene expression profiles for *sox2* in embryos chilled for 180 min at 0 °C in the presence of methanol. Following chilling, methanol was replaced with egg water and embryos were cultured at 27 ± 1 °C until hatching stage. Gene expression immediately after chilling and at the 20 somites, heartbeat and hatching stages was assessed using reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated 3 times. Results represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different concentrations of MeOH and non-chilled control within the same gene ($n = 9$).

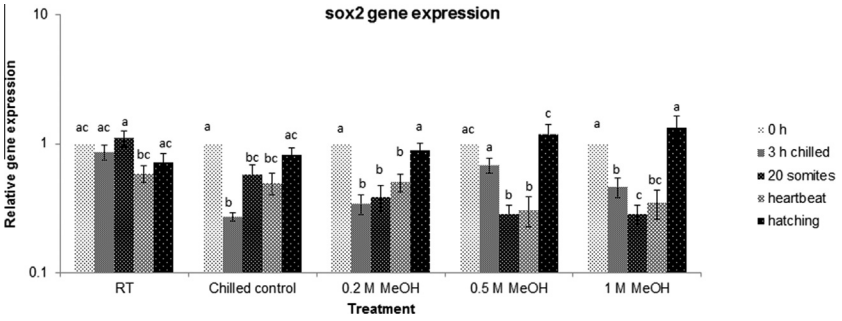

Fig. 2b. Effect of chilling in the presence of MeOH on *sox2* gene expression in zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox2* in embryos chilled for 180 min at 0 °C and then cultured at 27 ± 1 °C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated 3 times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different developmental stages of zebrafish embryos within same chilling treatment period ($n = 9$).

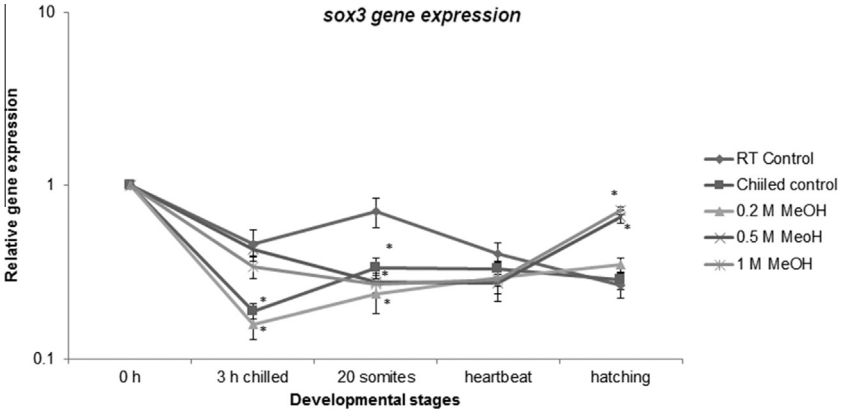

Fig. 3a. Effect of chilling and warming in different concentrations of MeOH on *sox3* gene expression in zebrafish embryos. The Figure shows the gene expression profiles for *sox3* in embryos chilled for 180 min at 0 °C in the presence of methanol. Following chilling, methanol was replaced with egg water and embryos were then cultured at 27 ± 1 °C until the hatching stage. Gene expression immediately after chilling and at 20 somites, heartbeat and hatching stages after culturing was assessed using reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated 3 times. Results represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different concentrations of MeOH and non-chilled control within the same gene ($n = 9$).

levels by the hatching stage. For the embryos that were chilled with 0.5 and 1 M MeOH, no significant decreases of *sox3* expression were observed until the 20 somites stage. The gene expression subsequently increased and was significantly higher than in non-chilled control embryos by the hatching stage.

4.3.2. Comparisons of sox3 gene expression at specific stages throughout development

In non-chilled control embryos, *sox3* (Fig. 3b) gene expression was decreased after 3 h and returned to control levels at the 20 somites stage before decreasing at the hatching stage. In the embryos that were chilled without MeOH, the levels of expression were decreased significantly immediately after chilling and then increased significantly following warming. Significant increases were observed at the hatching stage in the embryos that were chilled with MeOH.

4.4. Experiment 2.3: Impact of chilling and warming on sox19a gene expression in zebrafish (D. rerio) embryos in the presence of MeOH

4.4.1. Comparisons of different concentrations of MeOH on sox19a gene expression

In control embryos, *sox19a* (Fig. 4a) gene expression levels remained stable throughout developmental stages. Significant decreases in gene expression were observed in embryos that were chilled in egg water for 3 h before the expression returned to control levels by the 20 somites stage. For embryos that were chilled in 0.5 M and 1 M MeOH, significant increases of *sox19a* gene expression were observed after 3 h chilling when compared to non-chilled controls. The *sox19a* gene expression remained significantly above the non-chilled control levels throughout the tested developmental stages.

4.4.2. Comparisons of sox19a gene expression at specific stages throughout development

In non-chilled control embryos (Fig. 4b), expression of *sox19a* remained stable before decreasing by the 20 somites stage at 27 ± 1 °C. In the embryos that had been chilled at 0 °C in egg water and cultured at 27 ± 1 °C, significant decreases were observed after 3 h chilling and at the hatching stages when compared to time 0. In embryos that were chilled in 0.5 and 1 M MeOH, *sox19a* gene expression increased significantly immediately after 3 h chilling and then decreased to the time 0 level after culturing at 27 ± 1 °C to the hatching stage.

4.5. Experiment 3: Impact of chilling and warming on sox protein expression in zebrafish (D. rerio) embryos in the presence of MeOH

There is a strong relationship between gene and protein expression levels as protein is usually produced based on the information obtained from a specific gene or mRNA. However, sometimes this relationship could be altered due to various reasons: analytical variability of the measurement technology, post transcriptional mechanism affecting mRNA stability and protein degradation and timing differences between gene and protein expressions [39]. Furthermore, transcript levels detected in mRNA profiling clearly do not reflect all regulatory processes in the cell, as post-transcriptional processes altering the amount of active proteins, such as synthesis, processing and modification of proteins. Therefore, in addition to monitoring gene expression at the transcriptional level, analysis of the protein is also important for the understanding of the cellular, metabolic and regulatory networks in living organisms [30]. As studies on gene expression (mRNA level) do not provide information on protein translation, further studies were carried out to assess simultaneous protein expression of sox2 and sox19a after 3 h chilling at 0 °C and after warming and culturing at 27 ± 1 °C until hatching stage as altered patterns of gene expression were observed at different developmental stages.

4.5.1. Effect of 3 h chilling on Sox2 and Sox19a protein expression

Expression of Sox2 protein remained stable under all treatment conditions (Fig. 5b). Sox19a protein expression level remained stable in non-chilled controls and embryos chilled in egg water for 3 h at 0 °C. Significant increases in Sox19a protein expression were observed in the embryos that were chilled with 1 M MeOH for 3 h at 0 °C.

4.6. Effect of 3 h chilling and subsequent warming on Sox2 and Sox19a protein expression in hatching stage embryos

Sox2 protein expression (Fig. 6) decreased significantly in hatched embryos when compared to 50% epiboly stage. No significant differences were observed between non chilled embryos at the hatching stage and embryos chilled with or without 1 M MeOH and subsequently cultured until the hatching stage.

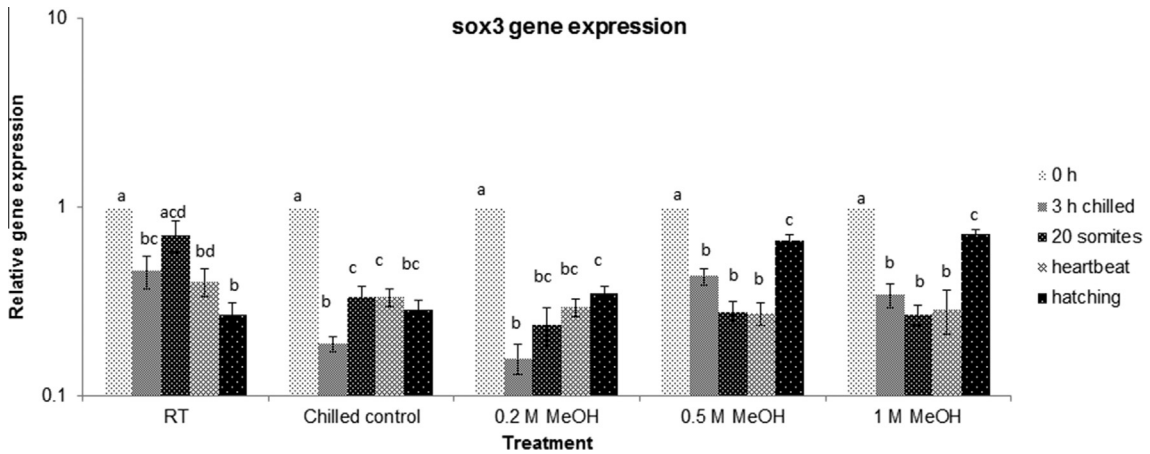

Fig. 3b. Effect of chilling in the presence of MeOH on *sox3* gene expression in zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox3* in embryos chilled for 180 min at 0 °C and then cultured at 27 ± 1 °C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated 3 times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different developmental stages of zebrafish embryos within the same chilling treatment period ($n = 9$).

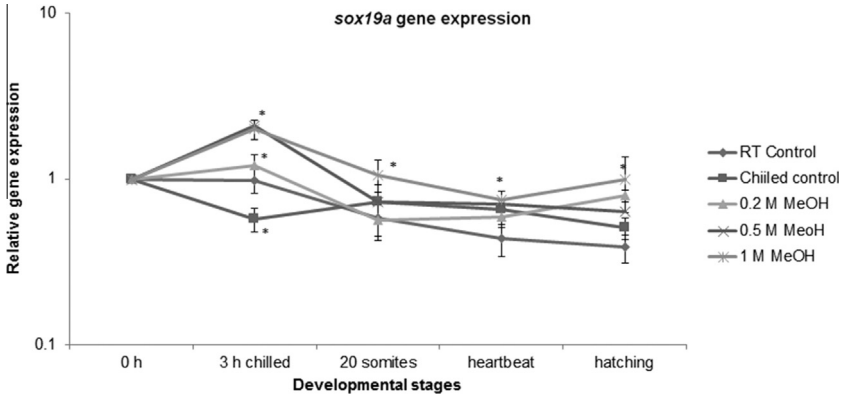

Fig. 4a. Effect of chilling in different concentrations of MeOH and warming on *sox19a* gene expression in zebrafish embryos. The Figure shows the gene expression profiles for *sox19a* in embryos chilled for 180 min at 0 °C in the presence of methanol. Following chilling, methanol was replaced with egg water and embryos were then cultured at 27 ± 1 °C until hatching stage. Gene expression immediately after chilling and at 20 somites, heartbeat and hatching stages was assessed using reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated 3 times. Results represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different concentrations of MeOH and non-chilled control within the same gene ($n = 9$).

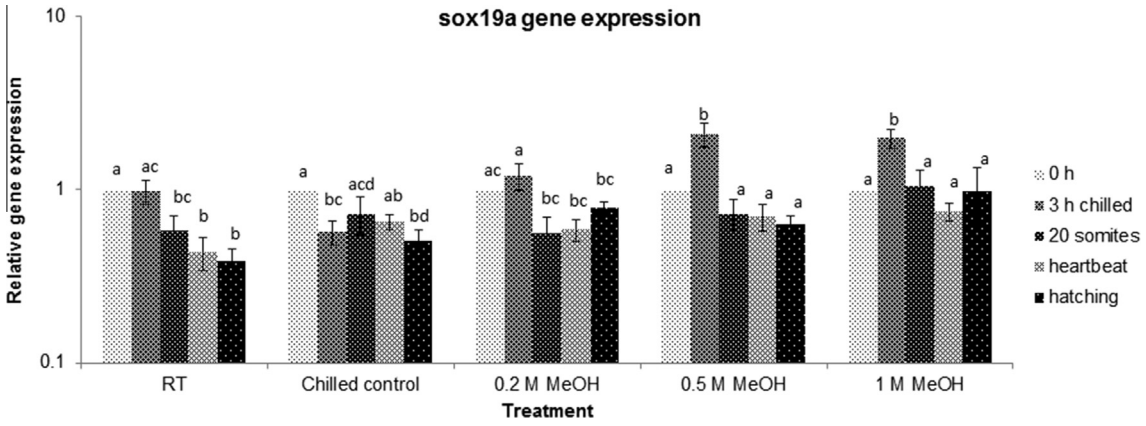

Fig. 4b. Effect of chilling in the presence of MeOH on *sox19a* gene expression zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox19a* in embryos chilled for 180 min at 0 °C and then cultured at 27 ± 1 °C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated 3 times. Points represent the mean expression levels relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different developmental stages of zebrafish embryos within the same chilling treatment period ($n = 9$).

Protein expression remained stable in both chilled and non-chilled embryos at the hatching stage. Protein expression of Sox19a remained stable from 50% epiboly stage to the hatching stages. No significant differences were observed in Sox19a protein expression between embryos that had been chilled with or without MeOH and non-chilled controls.

5. Discussion

Cryoprotectant toxicity studies are necessary before they are used in any chilling storage and cryopreservation protocol in order to minimise the effect of these chemicals. Survival rate has been widely used to assess cryoprotectant toxicity in embryos [20] and oocytes [35] prior to their chilling storage or cryopreservation. Methanol has been demonstrated to be an effective cryoprotectant in zebrafish embryo chilling storage [47] and oocyte cryopreservation [16]. However, there is no information available in the literature on the effect of methanol on gene and protein expression. This information is important due to the fact that MeOH is neurotoxic [7]. Studies have shown that methanol exposure leads to severe CNS defect in mice embryos [7] and drosophila embryos [26]. Therefore the aim of the present study was to investigate the effect of MeOH on gene and protein expression of zebrafish embryos following short term chilling and subsequent culture after warming.

5.1. Assessment of chilling tolerance of zebrafish embryos at 0 °C

An initial study was carried out to investigate chilling tolerance of 50% epiboly embryos for up to 24 h at 0 °C with the presence of different concentrations (0.2-,0.5- and 1 M) of MeOH. Results indicated that 50% epiboly stage embryos tolerated chilling for up to 6 h with/without MeOH (90 ± 5% survival rate) before it was significantly decreased after 18 and 24 h at 0 °C (18 and 24 h results, 10 ± 5% and 7 ± 5%, respectively). Previously, Zhang and Rawson demonstrated that over 50% of shield stage (~60% epiboly) embryos were killed when they were exposed to 0 °C for 4 h without any cryoprotectant and no embryo younger than bud stage survived 11 h exposure at 0 °C [49]. Due to the fact that zero or low survival rates were obtained for embryos chilled at 0 °C after 24 h, no longer term chilling studies in the presence of MeOH had been carried out with 50% epiboly stage embryos previously. Results obtained in medaka embryos [41] showed that gastrula stage embryo survival rate was not affected by 24 h chilling at 0 °C in hank's solution. In the present study, survival rate of similar stage 50% epiboly was reduced to 10% following chilling in egg

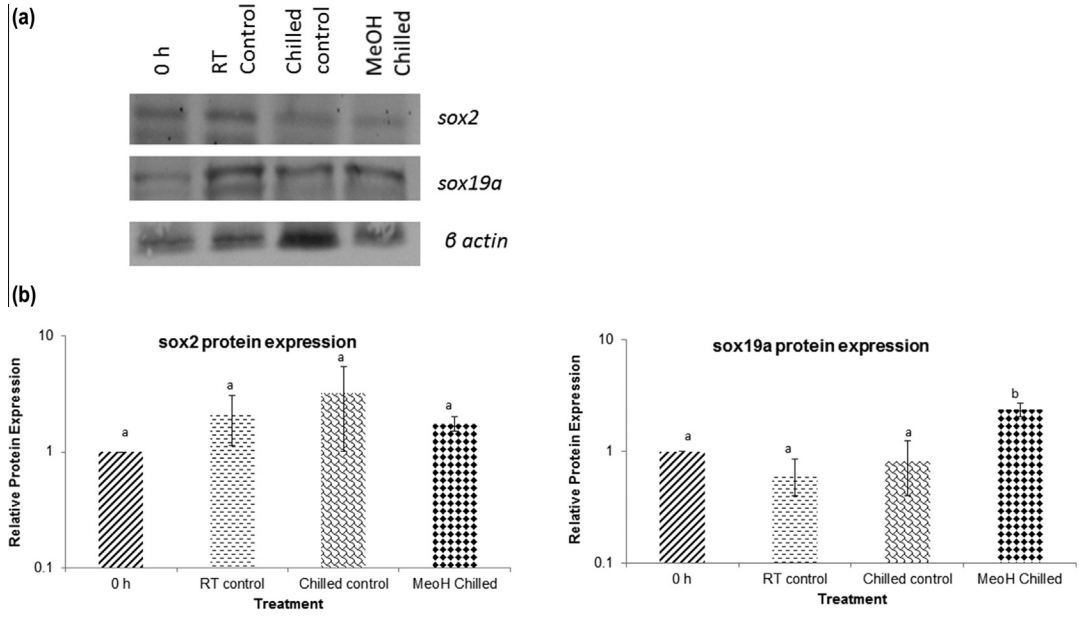

Fig. 5. Effect of 3 h chilling with or without the presence of MeOH on Sox2 and Sox19a protein expression in 50% epiboly zebrafish embryos. Protein expression profiles (b) are for Sox2 and Sox19a for embryos chilled for 180 min at 0 °C assessed by Western Blot (a). For each time point, 75 embryos were collected in triplicate and each experiment was repeated 3 times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos in post 3 h 50% epiboly stage ($n = 9$).

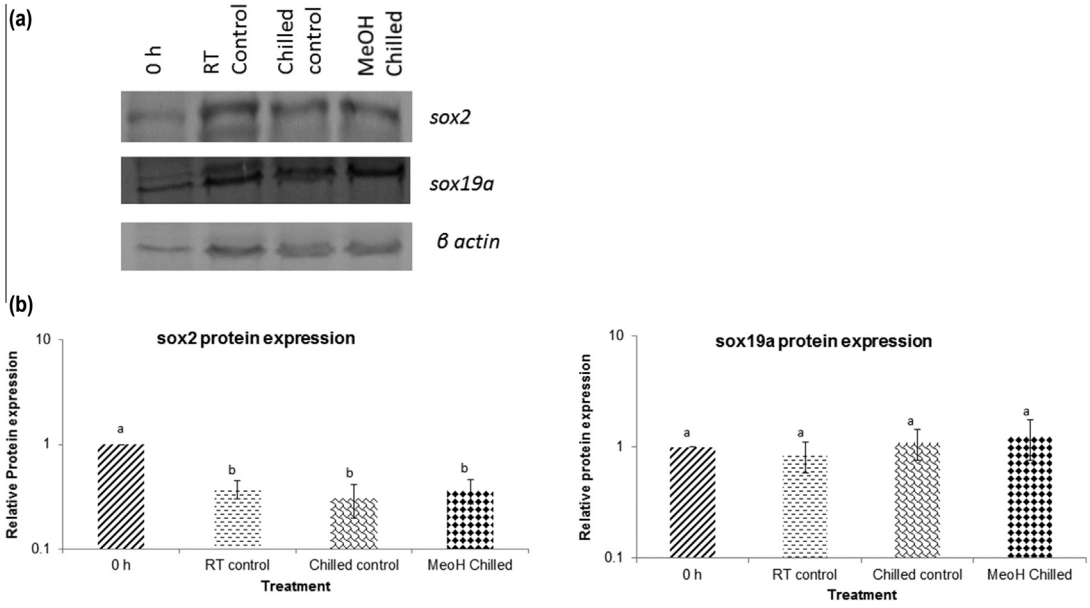

Fig. 6. Effect of 3 h chilling with or without the presence of MeOH and subsequent warming and culturing on Sox2 and Sox19a protein expression in hatching stage zebrafish embryos. Protein expression profiles (b) are for Sox2 and Sox19a in embryos chilled for 180 min at 0 °C and cultured at 27 ± 1 °C assessed by Western Blot (a). For each time point, 75 embryos were collected in triplicate and each experiment was repeated 3 times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos in hatching stage ($n = 9$).

water for 18 and 24 h at 0 °C. The difference is mainly species specific as medaka embryos are usually less susceptible to chilling [41]. Developmental of medaka embryos is slower (9 days to hatch) than zebrafish embryos (3 days to hatch). The reasons for the extent of stage-dependent chilling sensitivity might be related to the changes in cell and tissue types, number of cells, effectiveness of repair mechanisms, and enzymatic reactions. Changes in the size and structure of the yolk compartment and membrane structures might be among the key factors.

Cell membranes are generally highly permeable to methanol as Zhang *et al.* reported in zebrafish that methanol penetrates ovarian follicle at a rate comparable to the rate of water transport and therefore, incubation of cells in MeOH does not lead to osmotic stress [46]. In the present study, no significant differences found in embryos hatching rates that had been chilled in MeOH (at all tested concentrations) and egg water at 0 °C up to 24 h. This could be due to the fact that gastrula stage embryos are highly chilling sensitive and the concentrations of MeOH used in the present

study was not effective in protecting embryos from chilling injury. Under the similar conditions e.g. 24 h chilling at 0 °C, chilling sensitivity of heartbeat stage embryos was reduced significantly with the introduction of 1 M MeOH in chilling media [49]. The mechanism by which certain cryoprotective agents protect embryos from chilling injury has not been well understood. High chilling sensitivity in early stage embryos such as 50% epiboly stage is believed to be associated with the large amount of intraembryonic lipids [24]. Studies on partial removal of yolk on chilling sensitivity in zebrafish embryos showed that chilling injury following rapid cooling could be mitigated after partial removal of yolk at the prim-6 stage [24]. A study on chilling of porcine embryos also showed that the sensitivity of porcine embryos to chilling was related to their high lipid contents, embryos becoming tolerant to chilling when their lipid contents were reduced [28]. Lipid phase transition (LPT) in cell membranes are also responsible for chilling injury in mammalian sperm [11] and oocytes [3]. At the temperature around phase transition, chilled membranes lose fluidity and become leaky, which causes damage to cells [44].

5.2. Impact of 3 h chilling and warming on sox gene expression

Studies were carried out to investigate the effect of 3 h chilling and warming on *sox* gene expression at different embryo development stages after embryos were cultured at 27 ± 1 °C until the hatching stage. MeOH has been demonstrated to penetrate zebrafish embryo membrane [48] and be neurotoxic where its exposure leads to severe CNS defect to mice CNS at gastrulation periods [7] and in drosophila embryos [26]. Therefore, developmental stages were selected based on their morphology during development – 20 somites (early nervous system development), heartbeat (mid brain development – early touch reflexes) and hatching (first time exposure to external environment). These stages are key stages to study the effect of *sox* genes due to the fact that these genes play important roles in nervous system development in zebrafish embryos [6,27,42], any changes in these genes can have adverse effects on embryonic development.

Results from the present study showed decreased gene expression when compared to RT in full controls for all three genes (*sox2*, *sox3* and *sox19a*) in the embryos that had been chilled for 3 h at 0 °C without MeOH. In our experiments, a fast (~300 °C/min) chilling rate was used to chill embryos at 0 °C for 3 h. Damage to the lamina (a part of nuclear envelope) has been reported to affect the gene expression as the lamina also functions as a structural nuclear protein and regulator of gene expression [38]. It is possible that MeOH protects the lamina of the nuclear envelope during chilling and therefore reduces the chilling injury. Methanol has been reported to be an effective cryoprotectant during chilling storage of zebrafish embryos at zero and subzero temperatures [49]. Methanol was also found to improve survival rates for 50% epiboly stage carp (*Cyprinus carpio*) embryos when they were cooled to 4 or 0 °C [9]. In our study, MeOH was shown to protect gene expression following chilling at 0 °C for 3 h in the embryos that were chilled with different concentrations of MeOH and the protective effect was increased with increasing concentration as alterations in gene expression were less when compared to embryos that were chilled without MeOH. Zhang *et al.* also suggested that higher concentrations of MeOH treatment generally provided better embryo survival rates when embryos were cooled at a fast cooling rate of 300 °C/min [47]. Further studies are needed on the molecular mechanisms of the effectiveness of MeOH in protecting fish embryos from chilling injury.

Following chilling, embryos were warmed up and then cultured at 27 ± 1 °C to hatching stages, investigations were then carried out on the level of gene expression in 20 somites, heartbeat and hatching stages. Significant increase in *sox19a* was observed in the embryos that were chilled with 1 M MeOH. The increase in *sox19a* gene expression may be due to the activation of a compensatory mechanism. For example, a compensatory mechanism can be activated to prevent the loss of gene transcript in order to recover gene expression during chilling [14]. The decrease in *sox2* and *sox3* gene expression may therefore have been compensated by the stable higher levels of *sox19a* expression throughout. This could be the reason for unaffected embryo survival rates after 3 h chilling at 0 °C despite the decrease of *sox2* and *sox3* gene expression. All three *sox* genes are from same family B1 *sox* and functionally redundant [31]. Alteration in *sox* gene expression could have adverse implications on long term development of the embryos. A study in mice demonstrated that over expression of *sox* genes can be carcinogenic and induce a large number of tumour types [10]. Toxicity studies in zebrafish embryos also demonstrated that exposure of low concentrations of Perfluorooctane sulfonate (PFOS) induced upregulation of *pax8* genes (falls in the same group as sox gene) which leads to the induction of apoptosis genes in zebrafish embryos and larvae [37]. More long term studies are needed in order to investigate the adverse effects of MeOH on larvae and adult fish.

5.3. Subsequent impact of chilling and warming on Sox2 and Sox19a protein expression

Transcript levels detected in mRNA profiling do not reflect all regulatory processes in the cell as post-transcriptional processes also alter the amount of active proteins, such as synthesis, processing and modification of proteins [25]. Therefore, in addition to monitoring gene expression at the transcriptional level, analysis of the protein is equally important for the understanding of cellular, metabolic and regulatory networks in living organisms [30]. In embryos that had been chilled for 3 h at 0 °C, no significant differences in Sox2 protein expression were observed between embryos chilled for 3 h with or without MeOH and non-chilled controls despite decreased *sox2* gene expression. This could be explained by the repair mechanism of *sox2* gene transcript during post transcriptional processes, such as post transcription and translation modification to repair loss of *sox2* gene transcript. Studies in *hsp90* in parasite *Giardia* chilled for 20 min on ice demonstrated post transcriptional repair mechanism by mRNA *trans*-splicing [29]. Degradation or fragmentation of mRNA due to chilling could be repaired by a similar mechanism. During the mRNA splicing of *sox2*, the splicing junction carries hallmarks of classical *cis*-spliced introns, suggesting that regular splicing machinery may be sufficient for repair of the open reading frame. A complimentary sequence in the intron regions adjacent to the splice sites may assist in positioning two pre-mRNA for processing [29]. Damage to *sox2* transcripts due to chilling, could be processed by pre-mRNA and produce protein as normal resulting in the recovered/unaffected protein expression observed in these experiments. Sox19a protein expression remained significantly above the control level following 3 h chilling at 0 °C before decreasing to non-chilled control levels at the hatching stage. This protein expression pattern is similar to the pattern obtained in gene expression studies. To compensate, expressions of *sox19a* genes and subsequent proteins should be elevated in order to maintain physiological conditions and subsequent development due to their redundant function [15]. High protein levels of *sox19a* could also be explained by a compensation mechanism. It is clear from the present study that MeOH protected embryos at the molecular level during chilling and the protective effect was increased with increasing concentrations of MeOH. However, after warming and culturing of embryos until hatching stage, higher concentration (eg. 1 M MeOH) also altered the pattern of gene expression. Increased gene expression may be a compensatory response in order to recover the loss of mRNA transcript during chilling.

However, no significant differences were observed in protein expressions in the embryos that had been chilled at 0 °C for 3 h and warmed then cultured to hatching stage when compared to non-chilled controls. However the mechanisms associated with the effect of chilling and warming on gene and protein expressions require further investigation. In the present study, 3 h chilling period was studied and the results do not provide information on the effect of long term chilling on embryos in the presence of MeOH. More studies are needed to assess effect of long term chilling on gene and protein expression.

6. Conclusion

The effect of chilling in the presence of cryoprotectant methanol on gene expression in 50% epiboly zebrafish embryos is studied here for the first time. Results from embryo hatching rates studies showed that embryos could tolerate chilling for up to 6 h with/without MeOH. While gene expression of *sox* decreased significantly in embryos that had been chilled without cryoprotectant, the degree of decrease was less pronounced in embryo that chilled with MeOH. The level of gene expression decrease was less affected as the concentrations of MeOH increased. The results showed that MeOH could protect embryos from transcript degradation. However, higher concentration of MeOH (1 M MeOH) also altered pattern of *sox* gene expression when compared to controls at hatching stage. The present study showed that alteration in *sox* gene expression does not necessarily mean changes in protein expression as protein expression of Sox2 and Sox19a were stable in the embryos chilled with 1 M MeOH. This could be explained by the repair mechanisms of the embryos which involved post transcription and translation where loss of gene transcript could be repaired and resulted in normal protein expression. These results provided useful information in understanding the mechanisms associated with chilling injuries of zebrafish embryos.

Conflict of interest

None declared.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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